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ENHANCED PRODUCTION OF LIGNIN PEROXIDASE AND MANGANESE PEROXIDASE BY *PHANEROCHAETE CHRYSOSPORIUM* IN A SUBMERGED CULTURE FERMENTATION AND THEIR APPLICATION IN DECOLOURISATION OF DYES

by

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Thesis submitted in fulfilment of the requirements for the degree of Master of Science

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LIST OF SYMBOLS

%	Percentage
β	Beta
α	Alpha
ρ	Para
±	Plus minus
^{o}C	Degree celcius

LIST OF ABBREVIATION

$(NH_4)_6Mo_7O_{24}$	Ammonium heptamolybdate
$Al.K(SO_4)_2$	Aluminum potassium sulfate
ATCC	American type culture collection
ATP	Adenosine 5'-triphosphate
BOD	Biochemical oxygen demand
BPE	Bleach Plant Effluent
C	Carbon
$C_5H_{10}O_5$	Xylose
$C_6H_{12}O_6$	Glucose
$CaCl_2$	Calcium chloride
CO_2	Carbon dioxide
COD	Chemical oxygen demand
Conc.	Concentration
$CoSO_4$	Cobalt (II) sulphate
$CuSO_4$	Copper sulphate
DDT	1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane
DNA	Deoxyribonucleic acid
DS	Dissolved solids
EC	Enzyme commission
ECF	Elemental chlorine free
et al.	And all
$FeSO_4$	Ferrous sulphate
g/l	gram per litre

Glox	Glyoxal oxidase
H_2O	Water
H_2SO_4	Sulphuric acid
H_3BO_3	Boric acid
HCl	Hydrochloric acid
HMDS	Hexamethyldisilazane
i. e.	In other word
kda	Kilodalton
kg/U	Kilogram per unit
KH_2PO_4	Potassium dihydrogen phosphate
KOH	Potassium hydroxide
Lac	Laccase
lbs	Pounds
LiP	Lignin peroxidase
m^3/U	Metre cubic per unit
mg/l	Milligram per litre
$MgSO_4$	Magnesium sulphate
mm	Millimetre
mM	Millimolar
MnO_2	Manganese dioxide
MnP	Mangnanese peroxidase
$MnSO_4$	Manganese sulphate
mRNA	Messenger ribonucleic acid
mU/ml	Milliunit per millilitre
N	Nitrogen
-N=N-	Azo bonds

$Na_2C_4H_4O_6$	Sodium tartrate
Na_2CO_3	Sodium carbonate
Na_2HAsO_4	Disodium orthoarsenate
Na_2SO_4	Sodium sulphate
NaCl	Sodium chloride
NADH	Reduced form of nicotinamide adenine dinucleotide (NAD^+)
$NADP^+$	Phosphorylated form of NAD^+
NADPH	Reduced form of $NADP^+$
$NaHCO_3$	Sodium bicarbonate
$NaMoO_4$	Sodium molybdenum oxide
ND	Not determined
NG	No growth
$NH_4H_2PO_4$	Ammonium phosphate
nm	Nanometre
nmols	Nano mols
OH	Hydroxyl
P. chrysosporium	*Phanerochaete chrysosporium*
PAH	Polycyclic aromatic hydrocarbons
PCB	Polychlorinated biphenyls
PCP	Pentachlorophenol
pI	Isoelectric point
PKC	Palm kernel cake
ppm	Part per million
PVA	Polyvinyl alcohol
RNA	Ribonucleic acid
rpm	Rotation per minute

SEM	Scanning electron microscopy
sp.	Species
TCF	Totally chlorine free
TEM	Transmission electron microscopy
TMB	1,2,4,5-tetramethoxybenzene
TNT	2,4,6-trinitrotoluene
TSS	Total suspended solids
U	Unit
UV	Ultraviolet
v/v	Volume per volume
VA	Veratryl alcohol
$Valc^{+}$	Veratryl alcohol radical cation
w/v	Weight per volume
$ZnSO_4$	Zinc sulfate
µl	Microlitre
µm	Micrometre

PENINGKATAN PENGHASILAN ENZIM LIGNIN PEROKSIDASE DAN MANGAN PEROKSIDASE OLEH *PHANEROCHAETE CHRYSOSPORIUM* DALAM FERMENTASI KULTUR TENGGELAM DAN PENGGUNAANNYA DALAM PENYAHWARNAAN PENCELUP

ABSTRAK

Masalah utama dalam merawat air sisa buangan industri adalah untuk menghilangkan pencelup. Sesetengah teknik rawatan secara fizik dan kimia adalah berkesan dalam penyahwarnaan pencelup tetapi bahan-bahan sampingan yang bertoksik juga dihasilkan. Ini menyebabkan terjadinya masalah pencemaran sekunder yang berpunca daripada penggunaan bahan kimia yang banyak. Bagi menangani masalah ini, kajian ini dijalankan bertujuan untuk mendegradasikan pencelup secara teknik biologi dengan menggunakan enzim lignin peroksidase (LiP) dan mangan peroksidase (MnP) yang dihasilkan oleh kulat *Phanerochaete chrysosporium*. Kajian ini bermula dengan pengoptimuman penghasilan enzim secara fermentasi kultur tenggelam berskala makmal. Dalam kajian penghasilan MnP sahaja, pengoptimuman parameter-parameter yang utama menunjukkan peningkatan dalam penghasilan MnP sebanyak 175%. Aktiviti MnP yang maksima (3.00 mU/ml) diperolehi apabila kulat *P. chrysosporium* ditumbuhkan di dalam medium yang mengandungi 0.2% (b/i) D-xilosa, 0.4% (b/i) ekstrak yis, 2.0 mM ammonia dihidrogenfosfat, 1.0 mM $MnSO_4$, saiz inokulum 1.0% (i/i) daripada 6 x 10^6 spora/ml, pH awal medium 4.0, kadar goncangan 100 psm dan eraman pada suhu bilik (28 ± 2 ^oC). Sebaliknya, dalam kajian penghasilan LiP sahaja, peningkatan sebanyak 183% diperolehi selepas pengoptimuman. Aktiviti LiP yang maksima (4.30 mU/ml) dihasilkan dengan 0.1% (b/i) sukrosa, 0.6% (b/i) ekstrak yis, 2.0 mM ammonia dihidrogenfosfat, 0.4 mM alkohol veratril, saiz inokulum 1.0% (i/i) daripada 6 x 10^6 spora/ml, pH awal medium 4.5, eraman pada suhu bilik (28 ± 2 ^oC) dan kadar goncangan 150 psm. Dalam penghasilan MnP dan LiP secara kombinasi, aktiviti MnP (4.64 mU/ml) dan LiP (5.37 mU/ml) yang

maksima telah ditingkatkan sebanyak 744% dan 253%, masing-masing. Keadaan optima yang diperolehi ialah 0.1% (b/i) D-glukosa, 0.6% (b/i) ekstrak yis, 2.0 mM ammonia dihidrogenfosfat, 0.4 mM alkohol veratril, 1.0 mM MnSO4, saiz inokulum 1.0% (i/i) daripada 6 x 10^6 spora/ml, pH awal medium 4.5, eraman pada suhu bilik (28 ± 2 ^oC) dan kadar goncangan 150 psm. Dalam penyahwarnaan pencelup metelina biru pula, ia boleh berlaku melalui dua peringkat iaitu penyerapan oleh biomas kulat atau/dan pendegradasian oleh aktiviti enzim. Kadar penyahwarnaan yang tertinggi (87%) diperhatikan di dalam medium yang mengandungi penghasilan LiP dan MnP dalam kombinasi, diikuti oleh LiP (82%) dan MnP (57%). Kesimpulannya, penghasilan LiP dan MnP dalam kombinasi memberikan keupayaan penyahwarnaan metelina biru yang paling berkesan berbanding dengan penghasilan enzim-enzim itu secara berasingan.

ENHANCED PRODUCTION OF LIGNIN PEROXIDASE AND MANGANESE PEROXIDASE BY *PHANEROCHAETE CHRYSOSPORIUM* IN A SUBMERGED CULTURE FERMENTATION AND THEIR APPLICATION IN DECOLOURISATION OF DYES

ABSTRACT

The main problem when treating industrial wastewater is the removal of dyes. Some of the physical and chemical treatment techniques were effective but they might result in the production of toxic by-products and could also be the cause of a secondary pollution problem due to excessive chemical use. In order to overcome the drawbacks, the present work has focused on the biodegradation of dyes using lignin peroxidase (LiP) and manganese peroxidase (MnP) by *Phanerochaete chrysosporium*. The study was started with the optimization of enzymes production in the laboratory scale of submerged system. In the study of producing MnP only, the optimization of some governing parameters resulted in an increment of MnP about 175%. The optimal yield of MnP (3.00 mU/ml) was found to be produced under the conditions of 0.2% (w/v) of D-xylose, 0.4% (w/v) yeast extract, 2.0 mM ammonium dihydrogenphosphate, 1.0 mM of $MnSO_4$, 1.0% (v/v) of $6x10^6$ spore/ml as inoculum, initial pH of 4.0, cultivation temperature at room temperature (28 ± 2 ^oC) and agitation speed at 100 rpm. On the other hand, in the study for optimization of LiP production only, there was an increment about 183%, and the optimal yield of LiP (4.30 mU/ml) was produced under the conditions of 0.1% (w/v) of sucrose, 0.6% (w/v) yeast extract, 2.0 mM ammonium dihydrogenphosphate, 0.4 mM of veratryl alcohol, 1.0% (v/v) of $6x10^6$ spore/ml as inoculum, initial pH of 4.5, cultivation temperature at room temperature (28 ± 2 ^oC) and agitation speed at 150 rpm. In the study of optimizing MnP and LiP in combination, the optimal yield of MnP (4.64 mU/ml) and LiP (5.37 mU/ml) resulted in an increment approximately 744% and 253%, respectively, and the optimized conditions were 0.1% (w/v) of D-glucose, 0.6% (w/v) yeast extract, 2.0 mM ammonium dihydrogenphosphate,

0.4 mM of veratryl alcohol, 1.0 mM $MnSO_4$, 1.0% (v/v) of $6x10^6$ spore/ml as inoculum, initial pH of 4.5, cultivation temperature at room temperature (28 ± 2 ^oC) and agitation speed at 150 rpm. As for the decolourisation of methylene blue, it could be occurred through two stages, which were adsorption on the fungal biomass or/and enzymatic biodegradation. The medium with mixture of enzymes, in which both LiP and MnP were secreted together, achieved the highest decolourisation rate (87%), followed by LiP (82%) and MnP (57%) when the enzyme was produced separately. Therefore, the production of LiP and MnP in combination provided the most powerful effect of decolourisation of methylene blue compared to the production of each enzyme separately.

CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

1.0 Lignin

Plant cell wall material is composed of three important constituents: cellulose, lignin and hemicellulose, which are called lignocellulosic material. Approximately 95% of plant biomass is composed of lignocellulosic material. Lignocellulose typically contains 45% cellulose, 25-30% hemicellulose and 25% lignin (Pérez *et al.*, 2002). These three types of polymers are strongly intermeshed and chemically bonded by non-covalent forces cross-linkages. Figure 1.1 shows the configuration of wood tissues.

Lignin is the most abundant organic material on earth after cellulose. It is found in the secondary wall and middle lamella of higher plants (Darah and Ibrahim, 1996). Lignin is a naturally occurring substance produced by plants to strengthen their tissues. The cellulose walls of the wood become impregnated with lignin, a process called lignification, which greatly increases the strength and hardness of the cell and gives the necessary rigidity to the tree.

Generally, lignin is found in all vascular plants. It makes vegetable firm and tastes crunchy. What we called fiber in our food is actually lignin. It functions to regulate the transport of liquid in the living plant. Additionally, it enables trees to grow taller and compete for sunshine (McCrady, 1991).

1.1 Structure of lignin

Lignin is a structurally complex aromatic biopolymer. It is a highly branched and three dimensional amorphous heteropolymer, non-water soluble and optically inactive. It is substituted of phenylpropane units (a benzene ring with a tail of three carbons) joined together by different types of linkages (Orth *et al.*, 1991; Schoemaker and Piontek, 1996; Ohkuma *et al.*, 2001). The polymer is synthesized by the generation of

free radicals, which are released in the peroxidase-mediated dehydrogenation of three phenyl propionic alcohols: coniferyl alcohol (quaiacyl propanol), coumaryl alcohol (ρ-hydroxyphenylpropanol), and sinapyl alcohol (syringyl propanol). The final result of this polymerization is a heterogeneous structure whose basic units are linked by C-C and aryl-ether linkages, with arylglycerol-β-aryl ether (β-O-4) being the predominant structure (Ramachandra *et al.*, 1988; Pérez *et al.*, 2002). Figure 1.2 shows the structure of lignin from gymnosperms and Figure 1.3 shows the structure of the three lignin precursors.

Lignin is formed by removal of water from sugars to create aromatic structures through the phenylpropanoid pathway. This reaction is irreversible. Lignin polymers are cross-connected structures with molecular weights on the order of 15, 000 or more (McCrady, 1991).

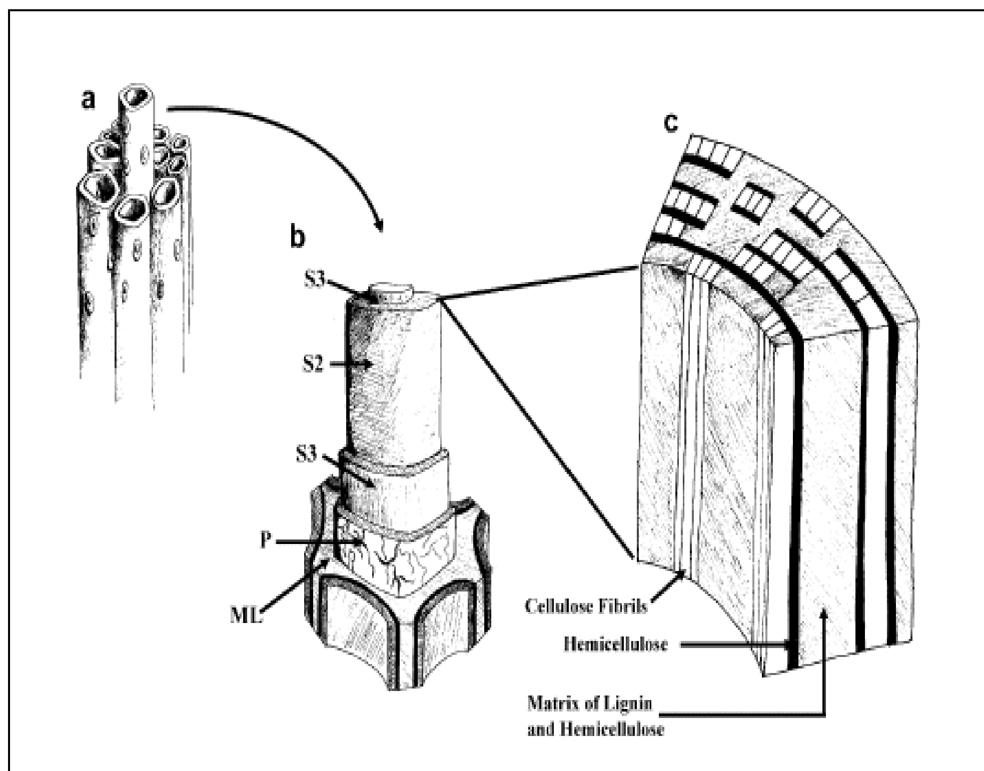

Figure 1.1: The configuration of wood tissues. (Pérez *et al.*, 2002)

a- adjacent cells; **b**- cell wall layers; S1, S2, S3- secondary cell wall layers; P-primary wall; ML-middle lamella; **c**- distribution of lignin, hemicellulose and cellulose in the secondary wall.

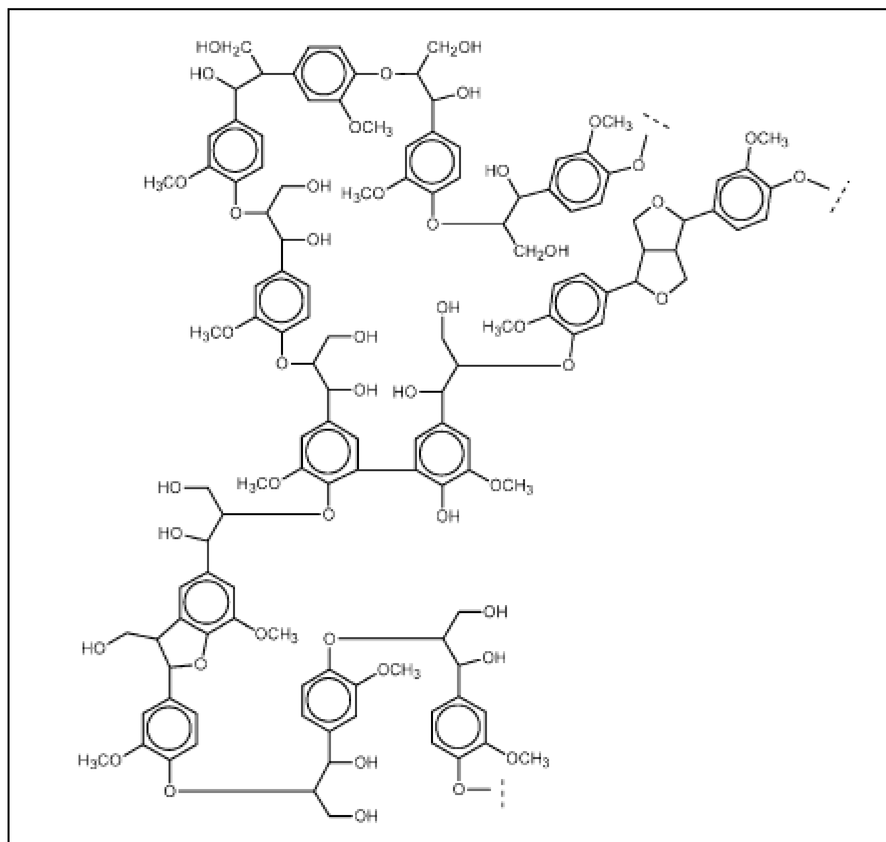

Figure 1.2: Lignin from gymnosperms showing the different linkages between the pheylpropane units. (Pérez *et al.*, 2002)

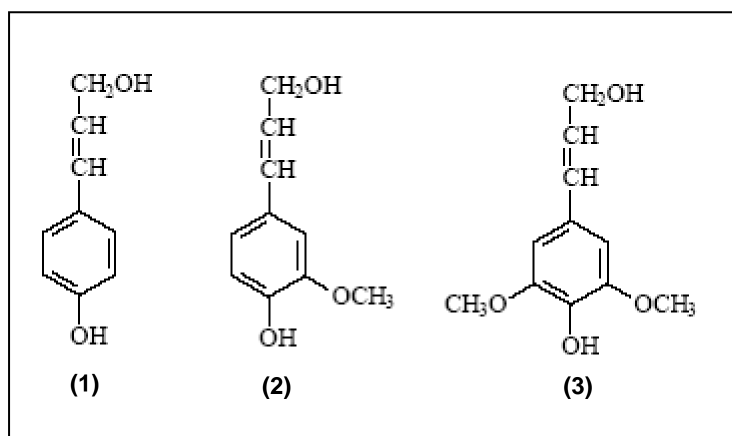

Figure 1.3: Structure of the three lignin precursors. (Haglund, 1999)

1- ρ-coumaryl alcohol; **2-** coniferyl alcohol; **3-** sinapyl alcohol

1.2 Lignin polymerization

After the polysaccharides have been deposited, lignin polymerization takes place in the cell walls. It is initiated by enzymatic oxidation of the precursors, which have been stated above, to form phenoxy radicals. These radicals can join with each other to become lignin polymer in numerous ways. Finally, the growing lignin polymer will form a complex cross-linked network (Figure 1.2; Haglund, 1999).

Figure 1.4 shows an early stage in the condensation of lignin precursors to form a lignin polymer (http://www.rpi.edu). The OH groups (either the alcoholic OH's on the chains or the phenolic OH's on the aromatic rings) can react with each other or with the aldehyde or ketone groups. An ether linkage is formed when an OH reacts with another. As shown in Figure 1.4, an OH reacts with an aldehyde to form a hemiacetal; an OH reacts with a ketone to form a ketal. There are several groups shown in red that can react further. Some will simply extend the polymer while others would establish cross linking. The monomer that is shaded in orange has three of its functional groups linked to other monomers, so it is starting a branch or cross link.

Figure 1.4: An early stage in the condensation of various monomers to form lignin (http://www.rpi.edu).

1.3 Lignin degradation

Lignin degradation is considered the rate-limiting steps in carbon cycling on the earth since there are only a few organisms capable of degrading this structurally complex aromatic biopolymer (Orth *et al.*, 1991).

The enzymes, those are extracellular, oxidative and unspecific, with the ability to liberate the highly unstable products which further undergo many different oxidative reactions, able to catalyze the initial steps of lignin depolymerization. Cleavage of C_α-C_β side chain bonds in lignin may be the most important reaction involved in its initial degradation (Ramachandra *et al.*, 1988; Pérez *et al.*, 2002).

Microorganisms that able to degrade lignin include the wood-rotting fungi and, to a lesser extent, certain actinomycetes and bacteria (Coll *et al.*, 1993). White-rot fungi are the most efficient degrader of wood lignin. Of these, *Phanerochaete chrysosporium* is the most extensively studied (Pérez *et al.*, 2002). These microorganisms produce ligninolytic enzymes, which consisted of two major families of enzymes: peroxidases and laccases. They have the ability to degrade the lignin, and effectively recycle plant biomass in the environment to CO_2 and H_2O. For catalizing such a reaction of lignin depolymerization, lignin peroxidase requires the presence of a C_α-hydroxyl group (Ramachandra *et al.*, 1988).

During lignin depolymerization, the chemical changes produced by white-rot fungi in lignin include oxidative cleavage of the propanoid side chains and also demethylation and oxidation cleavage of aromatic rings. Lignin biodegradation does not proceed by an orderly removal of the peripheral subunits. Instead it involves oxidation of the aromatic rings and side chains in the interior of the polymer, increasing the solubility of the polymer core and at the same time fragments of varying size are set free (Haglund, 1999).

According to Schoemaker and Piontek (1996), the lignified cell wall is progressively degraded from the inside outwards. In this process, fungal hyphae grow within the lumen of the woody cells, excreting both ligninolytic and (hemi-)cellulolytic

enzymes. Also, during ligninolysis, the fungi produce an extracellular mucilaginous sheath, closely associated both with the hyphae and with the decaying wood. At the early stages of decay, the ligninolytic enzymes, like lignin peroxidase (LiP), are found at the surface of the lignified wall, but it is unable to penetrate it. At later stages, LiP is found within the degraded regions. It is unclear if small, diffusible agents are necessary for the initial degradation.

Lignin is insoluble in water, and its degradation *in vivo* takes place in a polysaccharide gel. H_2O_2, the oxidant for LiP, is present in low concentrations in fungal cultures. It is to be expected that oxygen gas, although absolutely required for ligninolysis, will also suffer from diffusional limitations in the polysaccharide gel. In general, diffusion is limited in this system, especially for large molecules like enzymes and the cell wall constituents. Moreover, lignin is (in part) covalently bound to hemicelluloses. Furthermore, an important practical observation is the fact that right from the start of the depolymerization, initial breakdown products of lignin are assimilated by the fungus and oxidized to CO_2.

Fungal uptake of the initial breakdown products and further assimilation of the small fragments is important since it provides one of the basic mechanisms of lignin degradation. In the oxidative depolymerization process, phenolic compounds are formed and are proned to polymerize again. Manganese peroxidase (MnP) or laccases (in other classes of white-rot fungi), will convert the phenolic LiP breakdown products to form quinones, which are further metabolized in a process that most probably involves reduction to the corresponding hydroquinones. Other small fragments may have a ring-opened structure. Also, in the degradation process, glycolaldehyde is formed from the C_γ-fragment, which is a substrate for glyoxal oxidase (Glox), one of the H_2O_2-producing enzymes. Other H_2O_2-producing enzymes are part of the polysaccharide-degrading system of the fungus.

1.4 Lignin degrading microorganisms

Lignin is an insoluble polymer. Therefore, the initial steps in its biodegradation must be extracellular. Due to its hydrophobicity and complex random structure that lacked the regular hydrolysable bonds, lignin is poorly biodegraded by most microorganisms. The organism known to extensively degrade lignin is fungi and, to a lesser extent, certain actinomycetes and bacteria (Coll *et al.*, 1993; Mester and Field, 1998).

1.4.1 Fungi

1.4.1.1 Basic ecology and physiology of fungi

Fungi are a very heterogenous group, nevertheless the typical fungi have a range of features that separate them from other organisms. Three major groups of fungi are recognized: the *molds* (filamentous fungi), the *yeasts* (unicellular fungi), and the *mushrooms* (filamentous basidiomycetes). Table 1.1 shows the classification and major properties of fungi (Madigan *et al.*, 2000).

Table 1.1: Classification and major properties of fungi (Madigan *et al.*, 2000).

Group	Common name	Hyphae	Type of sexual spore	Habitats
Ascomycetes	Sac fungi	Septate	Ascospore	Soil, decaying plant material
Basidiomycetes	Club fungi, mushrooms	Septate	Basidiospore	Soil, decaying plant material
Zygomycetes	Bread molds	Coenocytic	Zygospore	Soil, decaying plant material
Oomycetes	Water molds	Coenocytic	Oospore	Aquatic
Deuteromycetes	Fungi imperfecti	Septate	None known	Soil, decaying plant material, surface of animal bodies

The habitats of fungi are quite diverse. Some are aquatic but most have terrestrial habitats, in soil or on dead plant matter, and these types often play crucial

roles in the mineralization of organic carbon in nature. A large number of fungi are parasites of terrestrial plants and a few fungi are parasitic on animals.

Fungi contain cell walls and produce spores. Fungal cell walls resemble plant cell walls architecturally, but not chemically. Although cellulose is present in the walls of certain fungi, many fungi have noncellulosic walls. Chitin, a polymer of the glucose derivative, N-acetylglucosamine, is a common constituent of fungal cell walls. Fungal cell walls are generally 80 – 90% polysaccharide, with proteins, lipids, polyphosphates, and inorganic ions that make up the wall-cementing matrix.

Most fungi are filamentous, which means that they consist of hyphae. The hyphae grow at their tips, and branch periodically, which creates a network of hyphae called mycelium. The mycelium arises because the individual hyphae form branches as they grow, and these branches intertwine, resulting in a compact mat. These hyphae can release digestive enzymes and take up nutrients over their entire length.

From the fungal mycelium, other hyphal branches may reach up into the air above the surface, and on these aerial branches spores called conidia are formed. Conidia are asexual spores, often highly pigmented and resistant to drying, and function in the dispersal of the fungus to new habitats. When conidia form, the white color of the mycelium changes, taking on the color of the conidia, which may be black, blue-green, red, yellow, or brown. Some fungi also produce sexual spores, which occur from the fusion either of unicellular gametes or of specialized hyphae called gametangia. Either an asexual or a sexual spore of a fungus can germinate and develop into a new hypha and mycelium.

Most of the fungi are heterotrophs, and due to their rigid cell wall they must excrete extracellular enzymes to break down complex polymers and then absorb simple nutrients (Haglund, 1999). A major ecological activity of many fungi, especially members of the Basidiomycetes, is the decomposition of wood, paper, cloth, and other products derived from natural sources. Basidiomycetes that attack these products are able to utilize cellulose or lignin from the product as carbon and energy sources. Lignin

is a complex polymer in which the building blocks are phenolic compounds. It is an important constituent of woody plants, and in association with cellulose it confers rigidity on them. The decomposition of lignin in nature occurs almost exclusively through the action of certain Basidiomycetes called wood-rotting fungi. Two types of wood rots are known: brown rot, in which the cellulose is attacked preferentially and the lignin left unchanged and white rot, in which both cellulose and lignin are decomposed. The white rot fungi are of considerable ecological interest because they play such an important role in decomposing woody material in forests.

When growth is restricted in some way, the intermediates of the primary metabolite pathway can be shunted over to other pathways which lead to a production of secondary metabolites. They differ in chemical composition and are often species- or strain-specific. Lignin is in most white-rot fungi degraded only during the secondary metabolism.

1.4.1.2 Degradation by fungi compared to bacteria

Bacteria are prokaryotes, unicellular organisms lacking a cellular nucleus as well as other organelles. Fungi are eukaryotes, and phylogenetically very distant from bacteria. In many features, their requirements for and means of growth and reproduction are also widely different. Normally, bacteria are fast growing compared to fungi and they can respond to a changing environment by populations utilizing the energy source present. However, there are some important advantages of using fungi instead of bacteria for biodegradation and decomposition of organic matter.

In the soil, there are high proportion of both fungi and bacteria being as decomposers. However, they degrade plant residues differently and have different roles in the recycling of nutrients. This is partly due to their different choice of habitats within the soil and the different types of organic matter they consume (http://www.soilhealth.segs.uwa.edu.au).

Fungi are generally much more efficient at assimilating and storing nutrients than bacteria. One reason for this higher carbon (C) storage by fungi lies in the chemical composition of their cell walls. They are composed of polymers of chitin and melanin, making them very resistant to degradation. Bacterial membranes, in comparison, are phospholipids, which are energy-rich. They degrade easily and quickly and function as a food source for a wide range of microorganisms.

The different proportions of C and N (i.e. different C:N ratios) of bacteria and fungi might also play a role in the mineralization and immobilization processes of nutrients in the soil. The C:N ratio for fungi varied between 7:1 and 25:1 due to their structure. Therefore, they need a greater amount of carbon to grow and reproduce. So, they will collect the required amount of carbon available from the soil organic matter. Bacteria, however, have a lower C:N ratio (between 5:1 and 7:1) and a higher nitrogen requirement and take more nitrogen from the soil for their own requirements.

Bacteria are suspended in or attached to a substrate while the fungal saprotrophs, which typically are filamentous, penetrate into the substrate with their hyphae. Fungi are recognized for their superior aptitudes to produce a large variety of extracellular proteins, organic acids and other metabolites, and for their capacities to adapt to severe environmental constraints. Moreover, fungi are robust organisms that have a high tolerance to toxic environments, making them ideal to use for bioremedial purposes. They can also withstand high temperatures and a wide range of pH, further enhancing their hardy capabilities (Lacina *et al.*, 2003; Mille-Lindblom, 2005). According to Haglund (1999), many of the pollutants are toxic to the organisms that are supposed to degrade them. The extracellular enzyme system of fungi enables them to tolerate considerably higher concentrations of a certain xenobiotica than bacteria that have their enzymes inside the cell. Also, many of these chemicals have low water solubility and are therefore not available to the same extent to bacteria. The nonspecific nature of the enzyme system enables the fungi to degrade complex mixtures of pollutants, such as the commercial PCB preparation Arochlor, all the way to

CO_2. This is very important, because there are always metabolites formed during the degradation that can be as toxic as or even more toxic than the original substance. In contrast, many different bacteria may be needed to successfully and completely degrade the same mixtures. The degrading system of fungi is usually induced by nutrient depletion and not by a particular pollutant. This is important because repression of enzyme synthesis does not occur when the concentration of a chemical is too low for effective enzyme induction. In this way the fungi can degrade very low concentrations of a pollutant and do not have to be preconditioned to it.

1.4.1.3 Ligninolytic fungi

Lignin degrading fungi or also known as ligninolytic fungi are classified into three major categories based on the type of wood decay caused by these organisms: white-rot fungi, brown-rot fungi, soft-rot fungi and litter-decomposing fungi (Haglund, 1999; Dhouib *et al.*, 2005). Among these three groups of fungi, white-rot fungi are the most effective lignin degraders and have been the most extensively studied group (Mester and Field, 1998; Ohkuma *et al.*, 2001). In addition, white rot fungi are the only known organisms capable of degrading lignin completely to CO_2 and H_2O (Buckley and Dobson, 1998).

White-rot fungi comprise a heterogeneous collection of several hundreds of species of basidiomycetes. Basidiomycetes, which cause white rot decay, are able to degrade lignin in wood. Lignin degradation by white rot fungi has been extensively studied, and results revealed that three kinds of extracellular ligninolytic enzymes, namely, lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (Lac), are responsible for initiating the depolymerization of lignin (Kondo *et al.*, 1994; Ohkuma *et al.*, 2001; Ikehata *et al.*, 2004).

In addition to lignin, white rot fungi are able to degrade a variety of environmentally persistent pollutants, such as a broad spectrum of structurally diverse aromatic compounds, including many xenobiotics, various polymeric dyes, polycyclic

aromatic hydrocarbons (PAH) and pulp bleach plant effluent (BPE) (Glenn and Gold, 1983; Michel *et al.*, 1991; Field *et al.*, 1992; Buckley and Dobson, 1998). Probably, this degradability of white rot fungi is due to the strong oxidative activity and the low substrate specificity of their ligninolytic enzymes. Thus, white rot fungi and their enzymes are thought to be useful not only in some industrial processes like biopulping and biobleaching but also in bioremediation and biotransformation (Ohkuma *et al.*, 2001).

Among the white-rot fungi, the most commonly used and best-studied model organism in lignin biodegradation studies is strains of *Phanerochaete chrysosporium* (Gold and Cheng, 1978; Orth *et al.*, 1993; Kondo *et al.*, 1994; Sundaramoorthy *et al.*, 1997; Kenealy and Dietrich, 2004).

1.4.1.4 *Phanerochaete chrysosporium*

Phanerochaete chrysosporium is the most extensively studied lignin-degrading white rot basidiomycete in which lignin peroxidase (LiP) and manganese peroxidase (MnP) were first discovered. It is a secondary decomposer of both hardwood and softwood branches and logs, and can be found in temperate forests throughout North America, Europe and Iran (Bonnarme and Jeffries, 1990; Orth *et al.*, 1991; Gill and Arora, 2003).

Phanerochaete chrysosporium, is a fungus that degrades wood. Since it looks like white chalk on rotting wood (Figure 1.5), it is called the white rot fungus. White rots are filamentous, or threadlike. The name derives from the bleached skeletal appearance of the crystalline cellulose left by selective degradation of lignin caused by these fungi (http://www.commtechlab.msu.edu; http://www.sciencedaily.com). It lives in fallen trees and on the forest floor.

Phanerochaete chrysosporium never form a fruiting body for reproduction, but form effused, very flat, fruiting bodies that appear as no more than a crust on the underside of a log. It is a member of the Basidiomycota, which means it bears its

meiotic spores externally on a structure called a basidium. Since *P. chrysosporium* produces asexual spores prolifically, it has distinct advantages over conidialess strains in its ease of manipulation (Gold and Cheng, 1978).

Figure 1.5: Growth of *Phanerochaete chrysosporium* on wood chips. (http://www.botit.batany.wisc.edu).

The crust for *Phanerochaete chrysosporium* comprises white to ochraceous-salmon colored basidiocarps often less than 0.25 mm thick that are interspersed over a thin mat of white monomitic hyphae. The effuse almost thin cobwebby appearance of this crust frequently makes this species difficult to spot in its natural habitat. *P. chrysosporium* displays both homothallic and heterothallic-bipolar sexuality. Microscopic examination of 2-week mycelial mats typically reveals simple septate hyphae ranging from 3-9 µm in diameter with sparse to moderate branching as well as the presence of thick-walled terminal or intercalary chlamydospores 50-60 µm in diameter. The blastoconidia are round to ellipsoid in appearance, 6-9 µm in diameter and are borne by poorly differentiated branched conidiophores (Figure 1.6) (https://www.fungalgenomics.concordia.ca).

Phanerochaete chrysosporium produce unique extracellular oxidative enzymes that degrade lignin. The lignin-degrading system of this fungus makes it very special.

Wood consists primarily of cellulose, which is white, and lignin, which is brown. In the lignin-degrading system, *P. chrysosporium* causes a white rot on wood because it decays the brown lignin and leaves the white cellulose behind. Therefore, it is collectively referred to as white rot fungi. *P. chrysosporium* has several features that might make it very useful. First, unlike some white rot fungi, it leaves the cellulose of the wood virtually untouched. Second, it has a very high optimum temperature (about 40°C), which means it can grow on wood chips in compost piles, which attain a very high temperature. These characteristics point to some possible roles in biotechnology.

Its ability in lignin-degrading system has generated much interest in the pulp and paper industry as an environmentally benign alternative to the chemical bleaching of pulp. In pulp and paper industry, one of the biggest energy expenditures in paper making comes from removal of the brown lignin from the wood so that the white cellulose is all that's left to make paper. By using the enzymes of a white rot fungus to remove the lignin, this could result in savings in both energy and time and avoid pollutive wastes being dumped out of the mills. The ideal fungus for this endeavor would be fast growing, able to tolerate high temperatures, and would leave the cellulose virtually untouched. This ideal fungus would have the exact characteristics of *Phanerochaete chrysosporium* (Michel, *et al.*, 1991; Kondo *et al.*, 1994; Moreira *et al.*, 1999).

Moreover, LiP and MnP enzymes isolated from *Phanerochaete chrysosporium* are useful in the bioremediation of a wide variety of organic waste byproducts including textile dyes, polyethylene, pesticides and herbicides, dynamite, PAHs, dioxins and oil-contaminated soil (Glenn and Gold, 1983; Field *et al.*, 1992; Orth *et al.*, 1993; Buckley and Dobson, 1998).

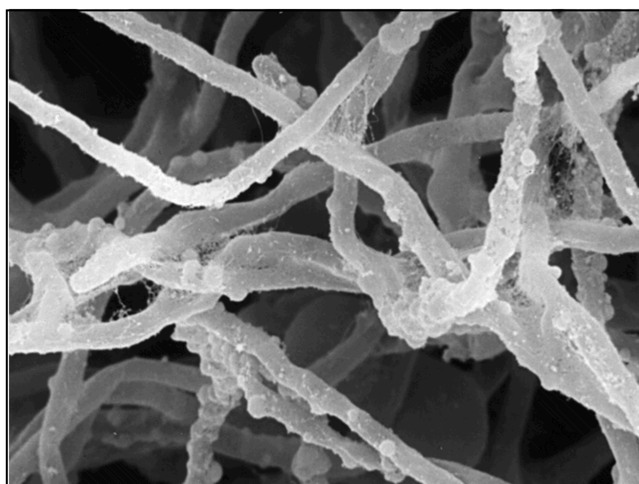

Figure 1.6: The cobweb-like appearance of *Phanerochaete chrysosporium*. (http://www.sciencedaily.com).

1.4.1.5 Other ligninolytic fungi

Some other fungi were also investigated for the production of extracellular peroxidases and phenoloxidase (laccase). Most of the researchers reported the investigation on the basidiomycete and only a few investigated on the ascomycete.

The fungi evaluated for the production of extracellular peroxidase and laccase are listed in Table 1.2.

Table 1.2: Fungi that produce peroxidases and laccase.

Microorganism	Type	Enzyme	Reference
Bjerkandera sp.	Basidiomycete	LiP, MnP	Mester and Field (1998); Teunissen and Field (1998); Moreira *et al.* (1999)
Ceriporiopsis subvermispora	Basidiomycete	LiP	Canales *et al.* (1998); Urzúa *et al.* (1998)
Chaetomium thermophilium	Ascomycete	Lac	Chefetz *et al.* (1998)
Chrysosporium lignorum	Basidiomycete	LiP, MnP	Buckley and Dobson (1998)
Coriolus hirsutus	Basidiomycete	MnP	Lacina *et al.* (2003)
Dichomitus squalens	Basidiomycete	MnP, Lac	Coll *et al.* (1993); Kondo *et al.* (1994); Gill and Arora (2003)
Elfvingia applanata	Basidiomycete	MnP, Lac	Ohkuma *et al.* (2001)
Flavodon flavus	Marine basidiomycete	MnP, Lac	Lacina *et al.* (2003)

Halosarpheia ratnagiriensis	Marine ascomycete	Lac	Lacina *et al*. (2003)
Irpex flavus	Basidiomycete	MnP	Gill and Arora (2003)
Irpex lacteus	Basidiomycete	MnP, Lac	Novotny *et al*. (2001)
Lentinus edodes	Basidiomycete	LiP, MnP, Lac	Bonnarme and Jeffries (1990); Kondo *et al*. (1994)
Lentinus squarrosulus	Basidiomycete	LiP	Wuyep *et al*. (2003)
Nematoloma frowardii	Basidiomycete	LiP, MnP	Hofrichter *et al*. (1999)
Oxyporus latemarginatus	Basidiomycete	LiP, MnP	Dhouib *et al*. (2005)
Phanerochaete chrysosporium	Basidiomycete	LiP, MnP, Lac	Faison and Kirk (1985); Bonnarme and Jeffries (1990); Michel *et al*. (1991); Orth *et al*. (1993); Haglund (1999); Rivela *et al*. (2000); Darah and Ibrahim (2001); Gill and Arora (2003); Lacina *et al*. (2003)
Phanerochaete crassa	Basidiomycete	MnP	Mariko *et al*. (2004)
Phanerochaete flavido alba	Basidiomycete	LiP, MnP	Bonnarme and Jeffries (1990); Lacina *et al*. (2003)
Phanerochaete magnoliae	Basidiomycete	LiP, MnP	Bonnarme and Jeffries (1990)
Phanerochaete sordida	Basidiomycete	LiP, MnP	Kondo *et al*. (1994); Moreira *et al*. (1999)
Phellinus pini	Basidiomycete	LiP, MnP	Bonnarme and Jeffries (1990)
Phlebia radiata	Basidiomycete	LiP, MnP, Lac	Bonnarme and Jeffries (1990); Coll *et al*. (1993); Hofrichter *et al*. (1999); Moreira *et al*. (1999)
Phlebia sp.	Basidiomycete	MnP, Lac	Dhouib *et al*. (2005)
Phlebia subserialis	Basidiomycete	MnP	Bonnarme and Jeffries (1990)
Phlebia tremellosa	Basidiomycete	MnP	Bonnarme and Jeffries (1990)
Pleurotus eryngii	Basidiomycete	MnP, Lac	Coll *et al*. (1993); Mester and Field (1998)
Pleurotus ostreatus	Basidiomycete	MnP, Lac	Coll *et al*. (1993); Mester and Field (1998)
Pleurotus sajor-caju	Basidiomycete	MnP, Lac	Novotny *et al*. (2001)
Poluporus sp.	Basidiomycete	LiP, MnP, Lac	Dhouib *et al*. (2005)
Polyporus sanguineus	Basidiomycete	MnP	Gill and Arora (2003)
Psathyrella atroumbonata	Basidiomycete	LiP	Wuyep *et al*. (2003)

Pycnoporus cinnabarinus	Basidiomycete	MnP, Lac	Novotny *et al.* (2001)
Rigidosporus lignosus	Basidiomycete	MnP, Lac	Coll *et al.* (1993)
Schizophyllum commune	Basidiomycete	Lac	Coll *et al.* (1993)
Sordaria fimicola	Ascomycete	Lac	Lacina *et al.* (2003)
Stereum annosum	Basidiomycete	Lac	Dhouib *et al.* (2005)
Stereum hirsutum	Basidiomycete	LiP, MnP, Lac	Kondo *et al.* (1994);Moreira *et al.* (1999)
Trametes (Coriolus or Polyporus) versicolor	Basidiomycete	LiP, MnP, Lac	Coll *et al.* (1993); Hofrichter *et al.* (1999); Moreira *et al.* (1999); Gill and Arora (2003); Lacina *et al.* (2003)
Trametes trogii	Basidiomycete	LiP, MnP, Lac	Haglund (1999); Dhouib *et al.* (2005)
Trichoderma atroviride	Ascomycete	Lac	Dhouib *et al.* (2005)

LiP: lignin peroxidase; MnP: manganese peroxidase; Lac: laccase

1.4.2 Ligninolytic bacteria

The decomposition of lignin in nature has been considered for a long time to occur by the action of wood-rot fungi mostly of the Basidiomycete class. These microorganisms simultaneously decompose lignin and wood polysaccharides. Nevertheless, several reports brought strong evidence of the ability of certain bacteria to degrade lignins.

Odier *et al.* (1981) reported the isolation of several bacterial strains able to degrade and assimilate isolated lignins. According to the results gained, there were eleven bacterial strains out of 122 soil isolates tested found to be able to grow by using poplar dioxane lignin as the sole carbon and energy source in mineral medium in aerobic conditions. In other words, these bacterial strains are able to produce ligninolytic enzyme to degrade lignin. The strains consisted of gram-negative aerobic rods identified as *Pseudomonas*, *Xanthomonas* and *Acinetobacter*.

According to Deschamps *et al.* (1980), some other lignin degrading bacteria were identified, including *Corynebacterium*, *Agrobacterium*, *Aeromonas*, *Klebsiella* and

Enterobacter. These strains were also able to assimilate different phenolic compounds considered as lignin related simple monomers.

In salt marsh ecosystems, lignin degradation is an important biogeochemical process due to the high primary productivity in such ecosystems and the abundance of vascular-plant-derived lignocellulosic material. Both bacteria and fungi can be involved in the lignin degradation. However, in aquatic environments, bacteria are probably responsible for the utilization of the most refractory components. In a salt marsh, bacteria mediate most of the degradation of lignin. One of the ligninolytic potential marine bacteria was *Sagittula stellate*, as reported by Gonzalez *et al*. (1997).

The ligninolytic bacterial strains are summarized in Table 1.3.

Table 1.3: Bacterial strains that involved in ligninolytic activity.

Strain of bacteria	**Reference**
Acinetobacter	Odier *et al*., 1981
Aeromonas	Deschamps *et al*., 1980
Agrobacterium	Deschamps *et al*., 1980
Corynebacterium	Deschamps *et al*., 1980
Enterobacter	Deschamps *et al*., 1980
Klebsiella	Deschamps *et al*., 1980
Pseudommonas	Odier *et al*., 1981
Sagittula	Gonzalez *et al*., 1997
Xanthomonas	Odier *et al*., 1981

1.4.3 Ligninolytic actinomycetes

The actinomycetes are a large group of filamentaous gram-positive bacteria that form branching filaments. As a result of successful growth and branching, a ramifying network of filaments is formed, called a mycelium. Although it is of bacterial dimensions, the mycelium is in some ways analogous to the mycelium formed by the filamentous fungi. Most actinomycetes form spores (Madigan *et al*., 2000).

Streptomyces are one of the genera in the actinomycetes. As the colony ages, streptomyces formed characteristic aerial filaments called sporophores, which project above the surface of the colony and give rise to spores. The spores called conidia. The conidia are produced simply by the formation of cross-walls in the multinucleate sporophores followed by separation of the individual cells directly into spores. Differences in the shape and arrangement of aerial filaments and spore-bearing structures of various species are among the fundamental features used in classifying the streptomyces groups. The conidia and sporophores are often pigmented and contribute a characteristic color to the mature colony (Madigan *et al.*, 2000).

The lignin-degrading actinomycete species examined to date have been shown to oxidatively depolymerize lignin. The primary degradative activity of actinomycetes is solubilization of lignin, with low levels of mineralization compared with the white rot fungi. The depolymerization reactions produce a modified water-soluble, acid-precipitable polymeric lignin as the principal lignin degradation product (Ramachandra *et al.*, 1988; Ball *et al.*, 1989; Pasti *et al.*, 1990). The range of actinomycete species capable of metabolizing lignin is still unknown. Moreover, the strains examined thus far solubilize lignin to an acid-precipitable polymeric lignin-like product. It has not been established whether species that are capable of more complete degradation of lignin (Pasti *et al.*, 1990).

On the other hands, Ramachandra *et al.* (1988) reported that the catabolism of lignin by actinomycete involves substantial initial cleavages of the lignin C_{α}-C_{β} and β-O-4 ether linkages, concomitant with other lignin oxidation reactions.

Many researches to date have been done with several strains of actinomycetes for their ligninolytic activity. Among the strains investigated, the strains that showed positive result in lignin degrading process included *Streptomyces* sp., *Amycolata* sp. and *Thermomonospora* sp.. The strains of actinomycetes that play a role in lignin degrading process are listed in Table 1.4.

Table 1.4: Actinomycetes that involved in ligninolytic activity.

Strain of actinomycetes	Reference
Amycolata autotrophica	Ball *et al.*, 1989
Streptomyces badius	Ball *et al.*, 1989
Streptomyces chromofuscus	Pasti *et al.*, 1990
Streptomyces cyaneus	Ball *et al.*, 1989; Pasti *et al.*, 1990
Streptomyces diastaticus	Pasti *et al.*, 1990
Streptomyces rochei	Pasti *et al.*, 1990
Streptomyces sp.	Crawford, 1978
Streptomyces viridosporus	Ramachandra et al., 1988
Thermomonospora mesophila	McCarthy *et al.*, 1986; Ball *et al.*, 1989

1.5 Ligninolytic enzymes

Microorganisms have evolved several of enzymes for degrading the different components of lignocellulosic material. These enzymes include cellulases (for degrading the cellulose), xylanases (for degrading the hemicellulose), and ligninolytic enzymes (for degrading the lignin).

There are two major families of ligninolytic enzymes which are involved in lignolysis: peroxidases and laccases (Ohkuma *et al.*, 2001; Sasaki *et al.*, 2001; Pérez *et al.*, 2002). These enzymes are capable of forming radicals inside the lignin polymer, which results in destabilization of bonds and finally in the breakdown of the macromolecule of lignin (Hofrichter *et al.*, 1999). The ligninolytic enzymes attack lignin directly and thereby are the most promising long term alternatives to lignin removal by physical and chemical processes.

1.5.1 Laccases

Laccases (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) are multicopper blue oxidase which are able to oxidize polyphenols with oxygen as final electrons acceptor. Moreover, they are also able to oxidize *ortho*- and aromatic amines by removing an electron and a proton from a hydroxyl group to form a free radical. Their

active site is constituted by four copper atoms and they utilize molecular oxygen as an oxidant for the oxidation of varieties of phenols and other aromatic compounds to corresponding reactive quinines (Coll *et al.*, 1993; Ikehata *et al.*, 2004).

The action mechanism of laccases, especially the role of the metalic center remains unknown. Two steps mechanism have been proposed: firstly, copper T1 extracts one electron from the substrate; secondly, electron is transferred to the T2 or T3 center. After complete reduction of the trinuclear center, the molecular oxygen reduction occurs. Schematic figure for the phenols oxidation by laccases are shown in Figure 1.7.

Laccases are widespread in nature; they have been found in many plants and fungal species. According to Coll *et al.* (1993), the laccase studied is a monomeric glycoprotein containing 6.5% carbohydrate and having a molecular weight of 64,000. It has an isoelectric point of 3.6. It is stable in a pH range from 3 to 9, and its optimum pH is 4.5.

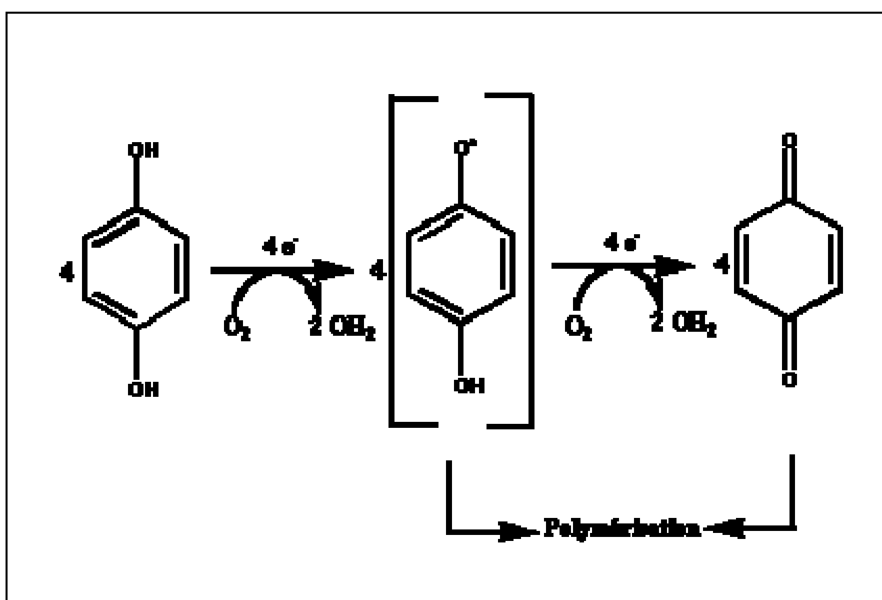

Figure 1.7: Phenol oxidation by laccases (http://www.lbs.fst).

Although the contribution of laccase to lignin degradation by white-rot fungi had long been speculated, its role in ligninolysis was less clear than those of lignin

peroxidase (LiP) and manganese peroxidase (MnP), partly because its low redox potential did not seem to be suitable for the oxidation of nonphenolic lignin structures (Ikehata *et al.*, 2004). Moreover, laccase has the capability of both polymerization and depolymerization of lignin model compounds (Haglund, 1999; Ikehata *et al.*, 2004), which made this issue more complicated.

1.5.2 Lignin peroxidase

Lignin peroxidase (LiP, ligninase, diarylpropane peroxidase; EC 1.11.1.14) is the first oxidative enzyme discovered in *Phanerochaete chrysosporium* (Glenn *et al.*, 1983; Tien and Kirk, 1983). LiP is an extracellular monomeric glycoprotein with a heme group in its active center. LiP has a molecular mass ranges from 38 to 43 kDa and pI from 3.3 to 4.7. It is capable of catalyzing the depolymerization of the aromatic polymer lignin and a variety of non-phenolic lignin model compounds in the presence of H_2O_2 (Teunissen and Field, 1998; Haglund, 1999; Pérez *et al.*, 2002; Ikehata *et al.*, 2004).

LiP has the distinction of being able to oxidize methoxylated aromatic rings without a free phenolic group (Figure 1.8), generating cation radicals that can react further by a variety of pathways, including nonstereospecific C_α-C_β cleavage and β-O-4 cleavage in lignin model dimers, aromatic ring opening, oxidation of benzyl alcohols such as veratryl alcohol to corresponding aldehydes or ketones, and hydroxylation of benzylic methylene groups (Darah and Ibrahim, 1996; Haglund, 1999; Ikehata *et al.*, 2004).

Ever since the discovery of LiP, veratryl alcohol has played a pivotal role in the study of the lignin biodegradation process. Veratryl alcohol is used as an assay for enzyme activity due to the easily detectable absorbance of the product veratraldehyde at 310 nm. LiP is capable of oxidizing non-phenolic compounds with a relatively high redox potential of the oxidized enzyme intermediates, lignin peroxidase Compound I (LiP I) or Compound II (LiP II) (Schoemaker and Piontek, 1996). Alternatively, it has been argued that the enzyme is capable of stabilizing the initial product of the veratryl

alcohol oxidation, the veratryl alcohol radical cation ($Valc^{+\cdot}$). Figure 1.9 shows the redox cycle of the enzyme schematically.

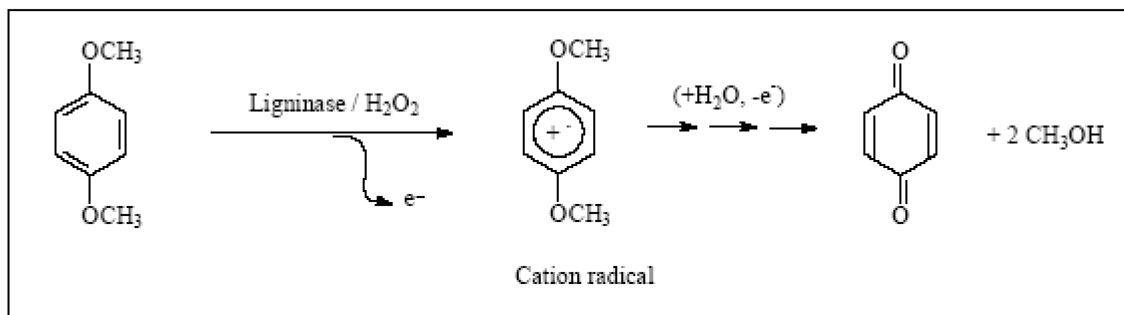

Figure 1.8: The action mechanism of generating cation radicals (Haglund, 1999).

The action mechanism of lignin peroxidase was discovered from studies of metoxylated benzenes. They were oxidized by the enzyme to unstable molecules called cation radicals. In the figure the cation radical 1,4-dimetoxibenzene, decomposes in the reaction with H_2O, producing methanol and benzenequinone.

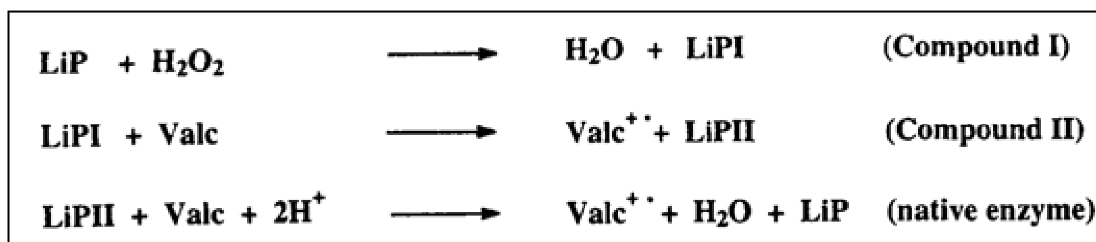

Figure 1.9: The lignin peroxidase redox cycle (Schoemaker and Piontek, 1996).

Thus, LiP I will oxidize the first molecule of veratryl alcohol to the corresponding radical cation ($Valc^{+\cdot}$), which is liberated from the active site. Subsequently, the second substrate molecule is oxidized by LiP II to form the second $Valc^{+\cdot}$. In the process, LiP II is converted to native enzyme. $Valc^{+\cdot}$ is a very strong oxidant, which subsequently might oxidize any recalcitrant chemical present, like the lignin polymer.

Formulation of the LiP redox cycle as depicted in Figure 1.9, in which electron transfer from veratryl alcohol to LiP I and LiP II, respectively, affords two $Valc^{+\cdot}$ species, makes the process amenable to a long-range electron transfer processes in the closely packed aromatic lignin polymer. The lignin polymer will bind to LiP and subsequently $Valc^{+\cdot}$ will serve as a continuous source of oxidant, provided of course that enough

H_2O_2 is generated. The electrons could tunnel through the lignin polymer from numerous sites, all with approximately the same destination, the binding site of the lignin polymer at the entrance to the active site channel (Schoemaker and Piontek, 1996).

1.5.3 Manganese peroxidase

Manganese peroxidase (MnP, manganese-dependent peroxidase; EC 1.11.1.13) was first discovered and purified from extracellular culture fluid of a basidiomycete *Phanerochaete chrysosporium* in the mid-1980s (Tien and Kirk, 1983; Glenn and Gold, 1985). The natural function of MnP is the degradation of the complex lignin polymer providing strength and rigidity to all higher plants. The enzyme catalyzes the H_2O_2-dependent oxidation of Mn(II) into highly reactive Mn(III). According to Sundaramoorthy *et al.* (1997), MnP is unique in its ability to catalyze the one-electron oxidation of Mn(II) to Mn(III) in a multi-step reaction cycle (Figure 1.10).

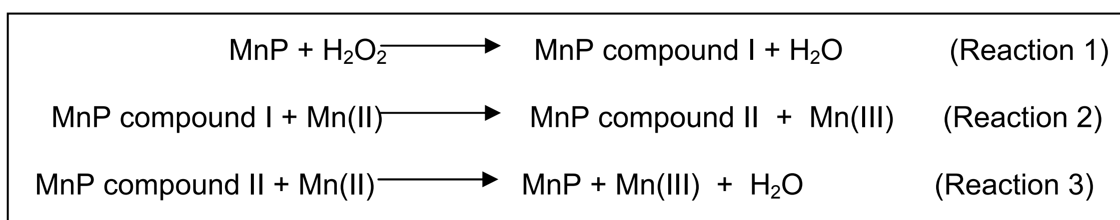

Figure 1.10: The oxidation of Mn(II) to Mn(III) (Sundaramoorthy *et al.*, 1997).

The enzyme-generated Mn(III) is complexed with a dicarboxylic acid such as oxalate, which is also secreted by the fungus. The Mn(III)-organic acid complex, in turn, oxidizes phenolic structures in various lignin related organic compounds, including vanillylacetone, 2,6-dimethyloxyphenol, curcumin, syringic acid, guaiacol, syringal dazine, divanillylacetone, and coniferyl alcohol, as well as organic dyes such as Poly-R, Poly-B, and phenol red (Tien and Kirk, 1983; Glenn and Gold, 1985; Darah and Ibrahim, 1996; Gill and Arora, 2003; Ikehata *et al.*, 2004; Mariko *et al.*, 2004). The action mechanism and catalytic cycle of MnP are shown in Figure 1.11 and 1.12, respectively.